

# Flavonoid content and antioxidant capacity of spinach genotypes determined by high-performance liquid chromatography/mass spectrometry

Mi Jin Cho,<sup>1</sup> Luke R Howard,<sup>1\*</sup> Ronald L Prior<sup>2</sup> and Teddy Morelock<sup>3</sup>

<sup>1</sup>Department of Food Science, University of Arkansas, 2650 N Young Avenue, Fayetteville, AR 72704, USA

<sup>2</sup>US Department of Agriculture, Agricultural Research Service, Arkansas Children's Nutrition Center, Little Rock, AR 72202, USA

<sup>3</sup>Horticulture Department, University of Arkansas, 316 Plant Science Building, Fayetteville, AR 72701, USA

## Abstract

**BACKGROUND:** Flavonoids in different spinach genotypes were separated, identified, and quantified by a high-performance liquid chromatographic method with photodiode array and mass spectrometric detection. The antioxidant capacities of the genotypes were also measured using two antioxidant assays—oxygen radical absorbance capacity (ORAC<sub>FL</sub>) and photochemiluminescence (PCL)—which measure the response to the peroxyl and superoxide anion radicals, respectively.

**RESULTS:** Eighteen flavonoids representing glucuronides and acylated di- and triglycosides of methylated and methylene dioxide derivatives of 6-oxygenated flavonols were identified (patuletin, spinacetin, spinatoside, jaceidin). The total flavonoids ranged from 1805 to 3703 mg kg<sup>-1</sup>, indicating 2.0-fold variation among genotypes. The ORAC<sub>FL</sub> and PCL values ranged from 48.7 to 84.4 mmol kg<sup>-1</sup> and from 9.0 to 14.0 mmol kg<sup>-1</sup>, respectively, representing as much as 1.7-fold variation among genotypes.

**CONCLUSION:** The ORAC<sub>FL</sub> and PCL values were highly correlated with total flavonoid content ( $r_{xy} = 0.96$ ).

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**Keywords:** spinach; flavonoids; antioxidant capacity; ORAC<sub>FL</sub>; PCL; HPLC/MS

## INTRODUCTION

Fruits and vegetables contain abundant amounts of antioxidant compounds, which are thought to be important in the maintenance of health and disease prevention.<sup>1</sup> Spinach (*Spinacia oleracea*) is an important dietary vegetable rich in antioxidants that is commonly consumed fresh in salads or after boiling fresh, frozen or canned leaves. Spinach contains several active antioxidant components, including flavonoids, *p*-coumaric acid derivatives, and uridine, which are reported to act synergistically.<sup>2</sup> Spinach leaves contain about 1000–1200 mg kg<sup>-1</sup> of total flavonoids,<sup>3,4</sup> and flavonoid levels have been shown to be affected by genetics,<sup>3,5</sup> maturation,<sup>6,7</sup> growing season,<sup>3</sup> fresh-cut processing and domestic cooking,<sup>4</sup> and frozen storage.<sup>5,8</sup> At least 15 flavonoids consisting mainly of patuletin and spinacetin derivatives have been identified in spinach. These include the glucuronides and acylated di- and triglycosides of methylated and methylene dioxide derivatives of 6-oxygenated flavonols (Fig. 1).<sup>3–5,9–12</sup>

Flavonoids are known to display a wide array of pharmacological and biochemical actions.<sup>13</sup>

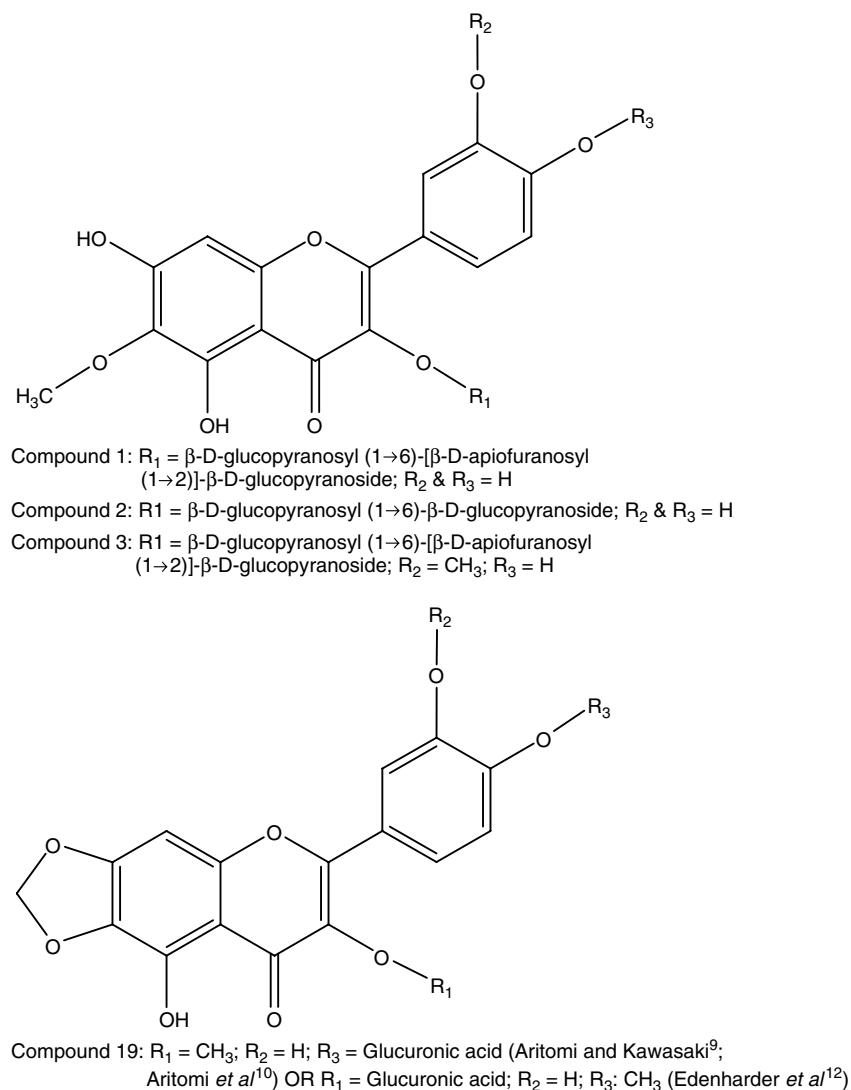
Flavonoids and other phenolic compounds act as antioxidants by the free radical scavenging properties of their hydroxyl groups, and are also effective metal chelators. The extensive conjugation across the flavonoid molecule and numerous hydroxyl groups enhances their antioxidant properties, allowing them to function as reducing agents, hydrogen or electron-donating agents, or free radical scavengers.<sup>14</sup> Flavonoids also possess anti-allergic, anti-inflammatory, anti-thrombotic, anticarcinogenic and antiviral actions, which in part may be related to their free radical scavenging properties.<sup>13</sup> Spinach ranks high among vegetables in oxygen radical absorbing capacity (ORAC), an *in vitro* assay that measures the peroxyl scavenging capacity of plant extracts.<sup>15</sup> In addition, spinach flavonoids and water-soluble spinach extracts have been shown to have antimutagenic,<sup>16,17</sup> antioxidative,<sup>2,18</sup> antitumor,<sup>19</sup> and anti-inflammatory properties<sup>20</sup> in biological systems, but have no potential adverse estrogenic activity<sup>21</sup> or toxic effects in animals.<sup>22</sup> These studies suggest that spinach extracts may exert beneficial effects such as chemo- and central

\* Correspondence to: Luke R Howard, Department of Food Science, University of Arkansas, 2650 N Young Avenue, Fayetteville, AR 72704, USA  
E-mail: lukeh@uark.edu

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**Figure 1.** Structures of the four major flavonoids in spinach.

nervous system protection, and anticancer and anti-aging functions.<sup>22</sup>

Although many flavonoids in spinach have been identified, we have consistently observed additional peaks absorbing strongly at 360 nm that have not been detected or identified in previous studies, indicating that the characterization of flavonoids in spinach is incomplete. Therefore, the objectives of this study were to establish a reverse-phase high-performance liquid chromatographic (HPLC) method with photodiode array (PDA) and mass spectrometric detection to separate, identify, and quantify flavonoid glycosides in selected spinach genotypes, and to examine the relationship between antioxidant activities and levels of total flavonoids.

## EXPERIMENTAL

### Chemicals

2,2'-Azobis(2-amidino-propane) dihydrochloride (AAPH) was obtained from Wako Chemicals, Inc. (Richmond, VA, USA), and trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid) was obtained

from Aldrich (Milwaukee, WI, USA). HPLC-grade methanol and acetonitrile were obtained from JT Baker Inc. (Phillipsburg, NJ, USA) and formic acid was obtained from Burdick & Jackson (Muskegon, MI, USA).

### Samples

Five genotypes of spinach were harvested at the mature stage and stored at  $-20^{\circ}\text{C}$  until analysis. Fallgreen, Samish, and F-380 are commercial cultivars, while 88-120 and 97-152 are advanced selections in the University of Arkansas breeding program.

### Analyses

#### Extraction

Frozen leaves were blended to a purée using a Black & Decker Handy Chopper Plus food processor. Subsamples (5 g) of purée were then homogenized for 1 min in 20 mL of extraction solution containing methanol–water–formic acid (60:37:3, v/v/v) using a Euro Turrax T18 Tissuemizer (Tekmar-Dohrman Corp., Mason, OH, USA). Filtered homogenates were centrifuged for 10 min at 5000 rpm. Aliquots

(4 mL) of supernatant were evaporated to dryness using a SpeedVac® (ThermoSavant, Holbrook, NY, USA) concentrator, with no radiant heat used during concentration, and resuspended in 1 mL of an aqueous 3% formic acid solution. All samples were passed through 0.45 µm filters (Whatman Inc., Florham Park, NJ, USA) prior to HPLC analysis. Triplicate extractions were prepared from each genotype.

#### HPLC analysis and purification of flavonoids

Flavonoids were analyzed using a Waters HPLC system equipped with a model 600 pump, model 717 plus autosampler, and model 996 photodiode array detector. Separation was carried out using a 4.6 mm × 250 mm Symmetry® C18 column (Waters Corp., Milford, MA, USA) preceded by a 3.9 mm × 20 mm Symmetry® C18 guard column. The mobile phase was a gradient of 0.1% aqueous formic acid (A) and acetonitrile–methanol–0.1% aqueous formic acid (7:2:1, v:v:v) (B), with 20–23% B for 12 min, 23–45% B for 31 min, and 45% for 2 min at 1.5 mL min<sup>-1</sup>. The system was equilibrated for 20 min at the initial gradient prior to each injection. A detection wavelength of 360 nm was used. External calibration curves were determined for patuletin-3-*O*-β-D-glucopyranosyl-(1 → 6)-[β-D-apiofuranosyl-(1 → 2)]-β-D-glucopyranoside (compound 1), spinacetin-3-*O*-β-D-glucopyranosyl-(1 → 6)-[β-D-apiofuranosyl-(1 → 2)]-β-D-glucopyranoside (compound 3), jaceidin-4'-β-D-glucuronide (compound 18), spinatocide-4'-*O*-glucuronide (compound 17), 5,3',4'-trihydroxy-3-methoxy-6:7-methylendioxyflavone-4'-β-D-glucuronide (compound 19), and 5,4'-dihydroxy-3,3'-dimethoxy-6:7-methylendioxyflavone-4'-β-D-glucuronide (compound 21), which were purified by semi-preparative HPLC using a SymmetryPrep™ C18 (7.8 mm × 300 mm) column and fraction collector II (Waters Corp). The gradient conditions were as follows: 20–23% B for 14.4 min; 23–45% B for 37.2 min; and 45% for 2 min, at a flow rate of 4.3 mL min<sup>-1</sup>. Other flavonoids were quantified as patuletin-3-*O*-β-D-glucopyranosyl-(1 → 6)-[β-D-apiofuranosyl-(1 → 2)]-β-D-glucopyranoside equivalents and expressed as mg kg<sup>-1</sup> fresh weight.

#### HPLC/MS–electrospray ionization analysis of flavonoids

An analytical Hewlett-Packard 1100 series HPLC instrument (Hewlett-Packard, Palo Alto, CA, USA) equipped with an autosampler, binary HPLC pump, and UV-visible detector was used following the same HPLC conditions described above. For HPLC/MS analysis, the HPLC apparatus was interfaced to a Bruker model Esquire LC/MS ion trap mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Mass spectral data were collected with the Bruker software, which also controlled the instrument and collected the signal. Typical conditions for mass spectral analysis in both positive and negative (to confirm molecular ion) ion electrospray modes included a capillary voltage of 4000 V, a nebulizing

pressure of 30.0 psi, a drying gas flow of 9.0 mL min<sup>-1</sup>, and a temperature of 300 °C. Data were collected using a full-scan mode over a mass range of *m/z* 50–1300 at 1.0 s per cycle. Characteristic ions were used for peak assignment (Table 1). Retention times were also used to confirm the identification of purified flavonoids.

#### Determination of antioxidant capacities

**ORAC assay.** Oxygen radical absorbing capacity (ORAC<sub>FL</sub>) of spinach extracts and purified flavonoids was measured using the method of Prior *et al.*<sup>23</sup> modified for use with a FLUOstar Optima (BMG Labtechnologies, Durham, NC, USA) microplate reader using fluorescein as fluorescent probe. Spinach extracts were diluted 600-fold with phosphate buffer (75 mmol L<sup>-1</sup>, pH 7) prior to ORAC analysis. The assay was carried out in clear 48-well plates (Falcon no. 3548). Each well had a final volume of 480 µL. Initially 40 µL of diluted sample, Trolox (TE) standards (6.25, 12.5, 25, 50 µmol L<sup>-1</sup>), and blank solution (75 mmol L<sup>-1</sup>, pH 7 phosphate buffer) were added to each well using an automatic pipette. The FLUOstar Optima plate reader equipped with two automated injectors was then programmed to add 400 µL fluorescein (0.108 µmol L<sup>-1</sup> stock), followed by 40 µL AAPH (99.1 mmol L<sup>-1</sup> stock) to each well. Fluorescence readings (excitation 485 nm, emission 520 nm) were recorded after the addition of fluorescein, after the addition of AAPH, and every 192 s thereafter for 112 min to reach a 95% loss of fluorescence. Final fluorescence measurements were expressed relative to the initial reading. Results were calculated based upon differences in areas under the fluorescein decay curve between the blank, samples, and standards. The standard curve was obtained by plotting the four concentrations of TE against the net area under the curve (AUC) of each standard. Final ORAC values of spinach extracts were calculated using the polynomial regression equation between TE concentration and the AUC, and are expressed as mmol of TE equivalents kg<sup>-1</sup> fresh weight.

**Photochemiluminescence (PCL) assay.** PCL assay using a Photochem® (Analytik Jena AG, Jena, Germany) instrument was used to measure antioxidant activity of spinach extracts and purified flavonoids against superoxide anion radicals that were generated from luminol, a photosensitizer, when exposed to UV light. An ACW (antioxidative capacity of water-soluble substances in aqueous buffer system) assay kit was obtained from the manufacturer and followed the protocol provided.<sup>24</sup> The antioxidant activity was estimated by the duration of lag phase, compared to TE standard curve, and expressed as mmol of TE equivalents kg<sup>-1</sup> fresh weight.

#### Statistical analysis

Analysis of variance<sup>25</sup> was used to determine significant differences (*P* < 0.05) in total flavonoid contents

**Table 1.** Peak assignment, retention time (RT), UV spectra, and mass spectral data of flavonoids detected in spinach

Peak	RT (min)	Identification	Spectral characteristics (nm)	M <sup>+</sup>	Fragments
1	6.8	Patuletin-3-O- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 6)-[ $\beta$ -D-apiofuranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucopyranoside	350.8, 257.0	788	333
2	9.9	Patuletin-3-O- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside	350.8, 257.0	657	333 (495)
3	10.5	Spinacetin-3-O- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 6)-[ $\beta$ -D-apiofuranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucopyranoside	353.2, 255.8	802	347 (671, 509)
4	12.6	Compound <b>6</b> isomer	347.2, 259.3	935	333 (615, 481, 463, 445, 427, 409, 371, 309)
5	13.3	Compound <b>7</b> isomer	347.2, 255.8	964	333
6	13.9	Patuletin-3-O- $\beta$ -D-(2''- $\rho$ -coumaroylglucopyranosyl)-(1 $\rightarrow$ 6)-[ $\beta$ -D-apiofuranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucopyranoside	317.4, 272.3	934	333 (603, 495, 441, 309, 291, 147, 119)
7	15.3	Patuletin-3-O- $\beta$ -D-(2''-feruloylglucopyranosyl)-(1 $\rightarrow$ 6)-[ $\beta$ -D-apiofuranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucopyranoside	336.5, 251.1	964	333
8	15.9	Compound <b>1</b> isomer	349.6, 257.0	788	333 (605, 495)
9	16.6	Spinacetin-3-O- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside	352.0, 255.8	671	347 (509)
10	19.0	Spinacetin-3-O- $\beta$ -D-(2''- $\rho$ -coumaroylglucopyranosyl)-(1 $\rightarrow$ 6)-[ $\beta$ -D-apiofuranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucopyranoside	317.4	948	347 (309, 177, 147, 119)
11	19.9	Spinacetin-3-O- $\beta$ -D-(2''-feruloylglucopyranosyl)-(1 $\rightarrow$ 6)-[ $\beta$ -D-apiofuranosyl-(1 $\rightarrow$ 2)]- $\beta$ -D-glucopyranoside	336.5	978	347 (633, 509, 471, 321, 177, 145)
12	20.5	Patuletin-3-O- $\beta$ -D-(2''- $\rho$ -coumaroylglucopyranosyl)-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside	317.4, 273.5	803	333 (309, 681, 177, 147, 119)
13	21.3	Patuletin-3-O- $\beta$ -D-(2''-feruloylglucopyranosyl)-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside	335.3, 251.1	832	333 (339, 501, 457, 177, 144)
14	21.9	Patuletin derivative	350.8, 271.2	801	331 (309, 693, 495, 177, 147, 199)
15	22.3	Patuletin derivative	341.3, 255.8	831	333 (693, 495)
16	26.1	Spinacetin-3-O- $\beta$ -D-(2''-feruloylglucopyranosyl)-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside	335.3	847	347 (509, 309, 177, 147, 199, 91)
17	29.4	Spinatocide	340.1, 270.0	523	347
18	32.2	Jaceidin-4'- $\beta$ -D-glucuronide	340.1, 271.2, 252.3	537	361
19	39.2	5,3',4'-Trihydroxy-3-methoxy-6:7-methylenedioxyflavone-4'- $\beta$ -D-glucuronide <sup>a</sup>	341.3, 277.1, 253.4	521	345
20	40.5	5,4'-Dihydroxy-3-methoxy-6:7-methylenedioxyflavone-4'- $\beta$ -D-glucuronide <sup>a</sup>	332.9, 277.1	505	329, 285
21	41.8	5,4'-Dihydroxy-3,3'-dimethoxy-6:7-methylenedioxyflavone-4'- $\beta$ -D-glucuronide <sup>a</sup>	341.3, 277.1	535	359

Patuletin: 3,5,7,3',4'-pentahydroxy-6-methoxyflavone.

Spinacetin: 3,5,7,4'-tetrahydroxy-6,3'-dimethoxyflavone.

Spinatocide: 3,6-dimethoxy-5,7,3',4'-tetrahydroxyflavone 4'- $\beta$ -D-glucuronide.

Jaceidin: 5,7,4'-trihydroxy-3,6,3'-trimethoxyflavone.

<sup>a</sup> Edenharter *et al.*<sup>12</sup> reports that glucuronide moiety is linked to the OH group at C3.

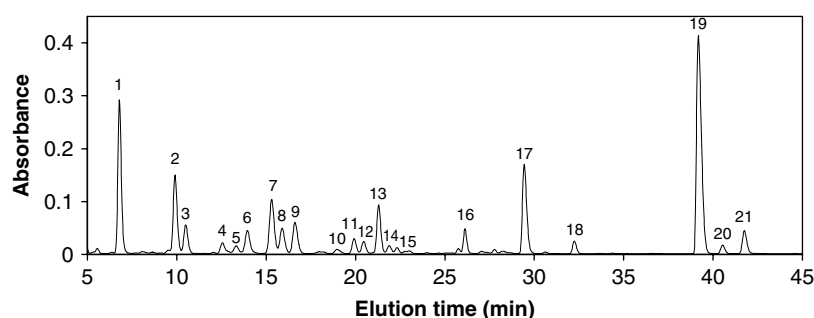
and antioxidant capacities among genotypes. The relationships between ORAC<sub>FL</sub> and PCL values and total flavonoid contents were determined using the Pearson correlation test.

## RESULTS AND DISCUSSION

### Identification of spinach flavonoids

Excellent separation of flavonoids in spinach extracts was achieved with the Symmetry<sup>®</sup> C18 column and gradient method applied. The HPLC profile of 21 flavonoids in the spinach genotype Fallgreen is shown in Fig. 2. Similar separation of flavonoids was obtained for the four other genotypes. The individual flavonoids

identified in the five spinach genotypes are listed in Table 1. Nineteen out of 21 flavonoids present were identified along with three unidentified flavonoids that contained patuletin aglycone (M<sup>+</sup> = 333). Previous studies detected 12 flavonoid compounds including glucuronides and acylated di- and triglycosides of methylated and methylene dioxide derivatives of 6-oxygenated flavonols. Aritomi and Kawasaki<sup>9</sup> identified the first three flavone glucuronides (compounds **18**, **19**, and **21**) in spinach leaves, and later detected four additional new flavonol glycosides (compounds **1**, **2**, **9**, and **17**).<sup>10</sup> Edenharter *et al.*<sup>12</sup> indicated that glucuronic acid is attached at C3, not C4' in the B-ring as identified by above authors. Ferreres *et al.*<sup>11</sup> identified



**Figure 2.** Detection of flavonoids (360 nm) in Fallgreen spinach. See Table 1 for peak identification.

five new flavonoid compounds – **3**, **7**, **10**, **11**, **16** – and confirmed the presence of compounds **1**, **9**, **18**, **19**, and **21** from spinach leaves. Using similar HPLC methodology, others<sup>3,4</sup> identified compounds **1**, **3**, **7**, **9**, **10**, **11**, **18**, **19**, and **21**. Their results concur with our findings regarding elution orders and mass spectral data, except that compound **9** (spinacetin-3-*O*- $\beta$ -D-glucopyranosyl-(1  $\rightarrow$  6)- $\beta$ -D-glucopyranoside) eluted earlier than compounds **10** and **11** (spinacetin-3-*O*- $\beta$ -D-(2''-*p*-coumaroylglucopyranosyl-(1  $\rightarrow$  6)-[ $\beta$ -D-apiofuranosyl-(1  $\rightarrow$  2)]- $\beta$ -D-glucopyranoside and spinacetin-3-*O*- $\beta$ -D-(2''-feruloylglucopyranosyl)-(1  $\rightarrow$  6)-[ $\beta$ -D-apiofuranosyl-(1  $\rightarrow$  2)]- $\beta$ -D-glucopyranoside). All genotypes contained 21 flavonoid components consisting mainly of 11 patuletin and five spinacetin derivatives. Flavonoids commonly found in other fruits and vegetables, such as flavonols (quercetin, myricetin, and kaempferol) and flavones (apigenin and luteolin) were not detected in spinach leaf extracts. In contrast to our results, kaempferol, quercetin, and myricetin were previously detected in fresh<sup>26–28</sup> and frozen spinach.<sup>29</sup> The flavonols in these studies were identified by comparison to authentic aglycone standards following acid hydrolysis, but no absorption spectra or mass spectral data were obtained. The mass spectral results of the flavonoids present in our samples clearly show that the flavonols quercetin, kaempferol, and myricetin, which have characteristic aglycone masses ( $M^+$ ) of 303, 287, and 320, respectively, were not present in the extracts. Hence we suspect that the identification of flavonols in spinach is incorrect. The HPLC profiles can clearly be used as a tool to differentiate variation in flavonoid components among spinach genotypes.

### Flavonoid composition and content

The contents of individual and total flavonoids of the five spinach genotypes are presented in Table 2. The content of total flavonoids ranged from a low of 1805 mg kg<sup>-1</sup> for Samish to a high of 3703 mg kg<sup>-1</sup> for 97–152, reflecting a twofold variation in total flavonoid content among genotypes. 88–120 (3168 mg kg<sup>-1</sup>) also contained high levels of total flavonoids compared to the commercial cultivars F-380 (2245 mg kg<sup>-1</sup>), Samish (1805 mg kg<sup>-1</sup>), and Fallgreen (2636 mg kg<sup>-1</sup>). The total flavonoid content of Samish (1805 mg kg<sup>-1</sup>) was similar to a previously reported value (1720 mg kg<sup>-1</sup>), but the total

flavonoid contents of 97–152 (3703 mg kg<sup>-1</sup>) and Fallgreen (2636 mg kg<sup>-1</sup>) were much higher than the previously reported values of 2241 and 1020 mg kg<sup>-1</sup>, respectively.<sup>3</sup> Other studies reported much lower levels (600–1500 mg kg<sup>-1</sup>) of total flavonoids in spinach.<sup>4,7</sup> The high levels of total flavonoids obtained in our study may be explained by the detection and quantification of numerous flavonoids that were not detected in previous studies. Interestingly, two of the advanced breeding selections, 97–152 and 88–120, had much higher levels of total flavonoids than the commercial cultivars, suggesting that selection for disease resistance in the breeding program has increased flavonoid content. The total flavonoid content of spinach genotypes compares favorably with other flavonoid-rich vegetables such as Swiss chard (2400–3000 mg kg<sup>-1</sup>)<sup>30</sup> and red onions (943 mg kg<sup>-1</sup>).<sup>31</sup>

The predominant flavonoids in all five genotypes were compound **1** (patuletin-3-*O*- $\beta$ -D-glucopyranosyl-(1  $\rightarrow$  6)-[ $\beta$ -D-apiofuranosyl-(1  $\rightarrow$  2)]- $\beta$ -D-glucopyranoside), compound **17** (spinatoside), and compound **19** (5,3',4'-trihydroxy-3-methoxy-6:7-methylenedioxyflavone-4'- $\beta$ -D-glucuronide). In addition to these compounds, the genotypes contained appreciable levels of other flavonoids. 88–120 was unique in that it contained the highest levels of compounds **2**, **9**, **11**, **13**, and **16**, and 97–152 had exceptionally high levels of compounds **6**, **7**, **13**, **17**, **19**, **20**, and **21** compared with the other genotypes. Howard *et al.*<sup>3</sup> did not detect compound **19** in the commercial cultivar Samish, which contrasts with our finding (398.8 mg kg<sup>-1</sup>). This discrepancy indicates that environmental growing conditions may affect the ability of specific spinach genotypes to synthesize individual flavonoids. In terms of composition, compound **19** was the predominant flavonoid in 97–152, Fallgreen, and Samish, whereas compound **1** was the predominant flavonoid in 88–120 and F-380. The levels of individual flavonoids were strongly affected by genotype, as reported previously.<sup>3,5</sup> The advanced breeding selections, 88–120 and 97–152, contained higher levels of compounds **1**, **2**, **4**, **5**, **6**, **7**, **11**, and **19** than the commercial cultivars, F-380, Fallgreen, and Samish. Additionally, the levels of compounds **3**, **9**, and **16** were the highest in 88–120, while the levels of compounds **12**, **14**, **17**, and **20** were the

**Table 2.** Flavonoid composition and content (mg kg<sup>-1</sup> fresh weight) of spinach genotypes

Compound	Genotype				
	380	88-120 <sup>a</sup>	97-152 <sup>a</sup>	Fallgreen	Samish
1	365.1 ± 16.6 <sup>b</sup>	646.3 ± 15.3	644.5 ± 45.8	438.7 ± 27.2	307.4 ± 11.2
2	236.2 ± 11.0	355.1 ± 16.0	348.8 ± 32.3	252.5 ± 14.0	169.4 ± 7.3
3	113.6 ± 5.1	180.5 ± 4.0	96.9 ± 5.9	58.8 ± 2.6	60.5 ± 2.8
4	29.5 ± 4.7	42.8 ± 2.0	70.0 ± 6.2	38.0 ± 5.0	24.2 ± 2.2
5	13.0 ± 4.6	31.0 ± 2.5	49.7 ± 2.6	25.1 ± 5.4	17.0 ± 1.8
6	68.5 ± 4.5	108.3 ± 10.8	165.2 ± 14.2	95.0 ± 4.5	48.7 ± 3.8
7	138.9 ± 5.1	252.5 ± 9.9	371.8 ± 14.5	211.4 ± 13.5	158.0 ± 6.9
8	30.5 ± 1.0	24.4 ± 2.1	68.3 ± 10.7	97.2 ± 6.1	25.4 ± 1.8
9	124.5 ± 6.5	183.7 ± 0.5	102.2 ± 9.1	76.0 ± 3.9	71.4 ± 4.2
10	21.3 ± 0.7	29.6 ± 4.0	25.3 ± 1.8	13.5 ± 0.6	10.9 ± 0.9
11	46.7 ± 2.6	95.8 ± 1.3	55.8 ± 2.6	31.7 ± 1.4	32.9 ± 1.7
12	36.0 ± 0.6	12.5 ± 4.0	60.1 ± 5.1	39.2 ± 1.2	21.4 ± 1.7
13	95.8 ± 5.2	142.6 ± 2.5	164.5 ± 15.0	156.3 ± 9.4	114.0 ± 4.3
14	25.1 ± 1.2	26.0 ± 0.6	32.2 ± 3.2	23.0 ± 1.0	12.6 ± 1.2
15	11.5 ± 1.2	17.0 ± 2.4	14.2 ± 0.6	14.6 ± 0.1	11.7 ± 1.0
16	50.4 ± 2.7	74.3 ± 2.1	45.1 ± 4.0	42.5 ± 2.5	41.4 ± 1.9
17	308.8 ± 16.6	287.2 ± 9.9	391.3 ± 30.7	326.6 ± 23.0	206.7 ± 2.6
18	105.7 ± 4.7	79.5 ± 3.5	88.9 ± 6.6	51.4 ± 3.2	33.4 ± 0.9
19	358.6 ± 19.0	503.7 ± 14.4	820.5 ± 52.0	592.1 ± 55.4	398.8 ± 3.8
20	4.3 ± 0.3	12.7 ± 0.3	19.6 ± 1.2	13.5 ± 1.4	7.2 ± 0.1
21	60.6 ± 3.3	62.6 ± 1.8	67.9 ± 4.0	38.7 ± 3.6	31.9 ± 0.6
Total <sup>c</sup>	2245d	3168b	3703a	2636c	1805e

<sup>a</sup> Breeding selection not available for sale or present in commerce at the time of this writing.

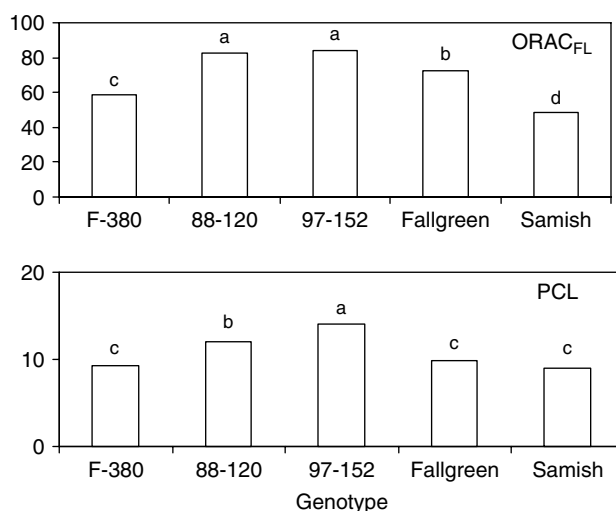
<sup>b</sup> Standard deviation ( $n = 3$ ).

<sup>c</sup> Values within rows with similar letters are not significantly different (LSD,  $P > 0.05$ ).

highest in 97–152. Howard *et al.*<sup>3</sup> also reported that advanced breeding lines of spinach had higher levels of several individual flavonoids (compounds **1**, **3**, **7**, **11**, **18**, and **19**) than commercial cultivars, indicating the effectiveness of disease resistance selection in producing flavonoid-enriched germplasm. Previous studies<sup>3,4</sup> detected no or low amounts of compound **16** (spinacetin-3-*O*- $\beta$ -D-(2''-feruloyl)glucopyranosyl)-(1  $\rightarrow$  6)- $\beta$ -D-glucopyranoside), but the levels of compound **16** ranged from a low of 41.4 mg kg<sup>-1</sup> for Samish to a high of 74.3 mg kg<sup>-1</sup> for 88–120 in our study.

#### Antioxidant activities against peroxy and superoxide anion radicals

The hydrophilic antioxidant capacities of spinach genotypes ranged from 48.7 to 84.4 mmol TE kg<sup>-1</sup> for ORAC<sub>FL</sub> and from 9.0 to 14.0 mmol TE kg<sup>-1</sup> for PCL (Fig. 3). 97–152 had the highest ORAC<sub>FL</sub> value (84.4 mmol TE kg<sup>-1</sup>), followed by 88–120 (82.7 mmol TE kg<sup>-1</sup>), Fallgreen (72.5 mmol TE kg<sup>-1</sup>), F-380 (58.9 mmol TE kg<sup>-1</sup>) and Samish (48.7 mmol TE kg<sup>-1</sup>), indicating a twofold variation in ORAC among the genotypes. A linear relationship was observed between ORAC<sub>FL</sub> values and total flavonoids ( $r_{xy} = 0.96$ ), indicating a major contribution of flavonoids to peroxy scavenging capacity. The ORAC<sub>FL</sub> values of all genotypes were much higher than the value of 22.2 mmol TE kg<sup>-1</sup> reported by Wu *et al.*<sup>15</sup> This discrepancy may be attributed to differences in genetics, maturation, and environmental



**Figure 3.** Antioxidant capacities (mmol TE kg<sup>-1</sup> fresh weight) of spinach genotypes. ORAC<sub>FL</sub>, oxygen radical absorbing capacity; PCL, photochemiluminescence assay. Bars within each graph with similar letters are not significantly different (LSD,  $P > 0.05$ ).

growing conditions. Howard *et al.*<sup>3</sup> reported that phenolic metabolism in spinach was significantly affected by both genetics and growing season, with leaves harvested in the spring having much higher levels of total phenolics and ORAC than leaves harvested in the fall. Leaf maturation can also markedly affect the ORAC of spinach. Mid-mature leaves were recently found to have 67% higher ORAC

values than immature and mature leaves, reflecting higher levels of total phenolics and flavonoids.<sup>6</sup>

The ranking of genotypes for PCL values followed the same order as ORAC<sub>FL</sub>; 97–152 (14.0 mmol TE kg<sup>-1</sup>) > 88–120 (12.0 mmol TE kg<sup>-1</sup>) > Fallgreen (9.8 mmol TE kg<sup>-1</sup>) > F-380 (9.3 mmol TE kg<sup>-1</sup>) > Samish (9.0 mmol TE kg<sup>-1</sup>). Consistent with results obtained for ORAC, a linear relationship was observed between PCL values and total flavonoids ( $r_{xy} = 0.96$ ), confirming a significant contribution of flavonoids to superoxide anion radical scavenging capacity. A linear relationship was also observed between ORAC<sub>FL</sub> and PCL values ( $r_{xy} = 0.87$ ), suggesting that flavonoids showed comparable ability to scavenge both peroxy and superoxide anion radicals. Our results are consistent with previous studies reporting that compounds in spinach possess high scavenging activities against a variety of free radical species. Using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, Gil *et al.*<sup>4</sup> reported that patuletin derivatives (compounds 1 and 7) possessing a 3',4'-dihydroxyl grouping had higher free radical scavenging activity against the DPPH radical than spinacetin derivatives (compounds 3, 9, 10, 11, and 16), and that acylation with ferulic acid (compounds 7, 11, 16) enhanced scavenging activity. Caldwell<sup>32</sup> measured the ORAC values of components of a spinach leaf extract separated by HPLC and found that several peaks absorbing optimally at 270 nm (phenolic acids), and many peaks absorbing optimally at 340 nm (flavonoids), exhibited peroxy radical scavenging activities. Bergman *et al.*<sup>18</sup> demonstrated that a glucuronated flavonoid (compound 19 in our study) and natural water-soluble antioxidants in spinach leaves were effective in scavenging several reactive oxygen species (O<sub>2</sub><sup>•-</sup>, OH<sup>•</sup>, <sup>1</sup>O<sub>2</sub>), which agrees with our finding (Table 3) that the compound scavenged both peroxy and superoxide anion radicals, albeit to a much greater extent for the peroxy radical. All three compounds had higher peroxy radical scavenging activity than ascorbic acid, whereas compound 1

showed higher superoxide anion radical scavenging activity than compounds 3 and 19, but greater than ascorbic acid. In contrast to our finding, a previous report<sup>4</sup> indicated that compound 19 had no nitrogen radical scavenging activity in the DPPH assay. This discrepancy may be due to differences in the chemical principles among the DPPH, ORAC, and PCL assays. The DPPH assay involves an electron transfer reaction, the ORAC assay reflects radical chain-breaking antioxidant activity by H atom transfer, while the complete reaction mechanism responsible for superoxide radical anion scavenging activity in the PCL assay is unknown.<sup>33,34</sup> The discrepancy in chemical structure proposed for compound 19 may also explain its radical scavenging capacity. According to Edenharder *et al.*<sup>12</sup> the glucuronide moiety on compound 19 is attached at the C3 position, whereas previous studies<sup>9,10</sup> report attachment at the 4' position on the B-ring. Blockage of the hydroxyl group at the 4' position is reported to greatly diminish radical scavenging activity;<sup>35</sup> hence if the glucuronide moiety is attached at the C3 position as proposed by Edenharder *et al.*<sup>12</sup>, the compound would still possess free radical scavenging capacity. Cao *et al.*<sup>36</sup> reported that spinach extracts effectively scavenged ROO<sup>•</sup> and OH<sup>•</sup> radicals, and also prevented oxidation induced by Cu<sup>2+</sup>. Our study confirms that spinach extracts possess significant peroxy and superoxide anion radical scavenging properties, and that flavonoids appear to be the major contributors to antioxidant capacity in spinach.

## CONCLUSION

The gradient method developed in conjunction with the Symmetry<sup>®</sup> C18 column allowed for baseline separation and identification of flavonoids in spinach genotypes. The levels of flavonoids, and ORAC<sub>FL</sub>, and PCL values varied twofold among genotypes, and genotypes with high levels of flavonoids exhibited the highest peroxy and superoxide anion radical scavenging capacities. The flavonoid and antioxidant-rich breeding selection, 97–152, appears to be an excellent candidate for commercial release, or may be exploited in future breeding efforts.

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**Table 3.** Antioxidant capacities (μmol TE μmol<sup>-1</sup>) of spinach flavonoids and ascorbic acid

Compound	ORAC <sub>FL</sub> <sup>a</sup>	PCL <sup>b</sup>
<b>1</b> , Patuletin-3-O-β-D-glucopyranosyl-(1 → 6)-[β-D-apiofuranosyl-(1 → 2)]-β-D-glucopyranoside	9.8a <sup>c</sup>	2.9b
<b>3</b> , Spinacetin-3-O-β-D-glucopyranosyl-(1 → 6)-[β-D-apiofuranosyl-(1 → 2)]-β-D-glucopyranoside	3.0b	0.03c
<b>19</b> , 5,3',4'-Trihydroxy-3-methoxy-6:7-methylenedioxyflavone-4'-β-D-glucuronide	6.4a	0.03c
Ascorbic acid	0.2c	0.8a

<sup>a</sup> ORAC<sub>FL</sub>, oxygen radical absorbing capacity.

<sup>b</sup> PCL, photochemiluminescence assay.

<sup>c</sup> Values within column with similar letters are not significantly different (LSD,  $P > 0.05$ ).

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